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Preventive effect of *tert*-butylhydroquinone on scrotal heat-induced damage in mouse testes

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ABSTRACT. To investigate the effect of *tert*-butylhydroquinone (tBHQ) on scrotal heat-induced damage in mice testes, 8-week-old mice were divided into 6 groups and administered with or without tBHQ through diet (10 mg/g), intraperitoneal injection (100 mg/kg body weight), or intratestis injection (12.5 mg/kg body weight), respectively. After single scrotal heat exposure (42°C for 25 min), trunk blood and testes were collected 48 h later. The testes from diet and intraperitoneal tBHQ-treated mice showed more compact interstitial cells and less germ cell loss in the seminiferous epithelium compared with their corresponding non-tBHQ groups. However, intratestis tBHQ treatment showed no marked difference relative to the non-treatment group. In addition, pre-treatment of tBHQ caused lower testosterone concentrations and reduced expression of

cytochrome P450 17α-hydroxylase/17,20-lyase (CYP 17) compared to the corresponding non-tBHQ groups. The results indicated that scrotal heat-induced structural damage was partly prevented by pre-treatment of tBHQ, which could be used as an effective antioxidant for preventing scrotal heat-mediated male infertility.

Key words: Scrotal heat; *Tert*-butylhydroquinone; Testis; Testosterone; 17α-hydroxylase/17,20-lyase

INTRODUCTION

Mammals have their testes suspended outside of the body cavity in order to maintain a lower testicular temperature than core body temperature (Hansen, 2009). Testicular temperature has been shown to increase in men who work in high ambient temperatures, such as bakers, welders, and drivers (Thonneau et al., 1998), and in animals exposed to high ambient temperatures, such as boars, mice, rats, and rams (Setchell, 1998; Li et al., 2013). Previous studies have also reported that scrotal temperatures above the normal range caused oxidative stress and germ cell loss in testes, which consequently results in male subfertility (Paul et al., 2009; Zhang et al., 2012). Therefore, there is increasing concern as to how to solve or prevent the problem of heat-induced sterility.

Tert-butylhydroquinone (tBHQ) is a synthetic phenolic antioxidant (Kim et al., 2009) that was approved for human use by both the Food and Agriculture Organization and the World Health Organization (1999). Recent studies have demonstrated that treatment with tBHQ up-regulated several enzymatic antioxidants associated with resistance against cisplatin-induced nephrotoxicity in rats (Perez-Rojas et al., 2011) and paraquat-induced dopaminergic cell degeneration in mice (Li et al., 2012). Furthermore, tBHQ has become a compound that is widely used as food antioxidants due to its easy addition in therapy and relatively low costs for patients. However, it is unclear whether tBHQ may protect testes from scrotal heat-induced damage.

Cytochrome P450 17α-hydroxylase/17,20-lyase (CYP 17), a microsomal enzyme catalyzing two distinct activities (17α-hydroxylase and 17,20-lyase), is critical in determining the production of androgens and estrogens in vertebrates (Liu et al., 2005; Chen et al., 2010). The 17α-hydroxylase steroid converts pregnenolone and progesterone to 17-hydroxylated steroids, which may be converted by 17,20-lyase to dehydroepiandrosterone and androstenedione, respectively (Chung et al., 1987). These latter two steroids are precursors of testosterone, which is a necessary prerequisite for the maintenance of established spermatogenesis in adult testes (Zirkin, 1998). Thus, normal testis function can be indicated by CYP 17 activity and testosterone concentration.

In the present study, a single scrotal heat model was used to investigate whether the antioxidant properties of tBHQ may prevent testicular heat-induced damage in scrotal heat mice administrated with tBHQ through diet, intraperitoneal (*ip*) injection, or intratestis (*it*) injection. In order to interpret the impact of tBHQ on steroid synthesis in testes, testosterone concentration in serum was determined, and testicular CYP 17 was located by immunohistochemistry.

MATERIAL AND METHODS

Animals

Eight-week-old adult male mice of the Institute of Cancer Research (ICR) were purchased from CLER, Japan, Inc. (Tokyo). Animals were housed in hanging stainless steel cages in a controlled environment of 25°C and 50 ± 5% humidity under a 12-h light/dark cycle. The animals were provided with food and water *ad libitum*. All experimental protocols were performed in accordance with the animal welfare regulations of the Tokyo University of Agriculture and Technology.

Experimental design

Eighteen mice were randomly divided into 6 groups: 1) diet-tBHQ (-), mice received basal diet for 1 week and were then exposed to scrotal heat, 2) diet-tBHQ (+), mice received a 10 mg/g tBHQ diet for 1 week and were then treated with scrotal heat, 3) *ip*-tBHQ (-), mice received a single *ip* injection of 10 mL/kg vehicle (saline solution) and were treated with scrotal heat 3 h later, 4) *ip*-tBHQ (+), mice received a single *ip* injection of 100 mg/kg body weight tBHQ and were treated with scrotal heat 3 h later, 5) *it*-tBHQ (-), mice received a single *it* injection of 10 µL/testis vehicle (saline solution), and 6) *it*-tBHQ (+), mice received a single *it* injection of 12.5 mg/kg tBHQ, which was previously dissolved in vehicle. Before *it* injection, mice were anesthetized first and then immediately exposed to scrotal heat.

All mice were subjected to a single scrotal heat exposure of 42°C for 25 min. Each animal was anesthetized by *ip* injection with 70 µg/g body weight chloral hydrate and the lower third of the body (hind legs, tail, and scrotum) was submerged in a water bath. After 25 min, each animal was dried and returned to its cage. Animals were sacrificed 48 h after scrotal heat exposure. Blood samples were collected following sacrifice and centrifuged at 3000 *g* for 10 min. The isolated serum samples were stored at -80°C until tested. Right testes from each animal were immersed in 4% paraformaldehyde solution for testicular histological and immunohistochemical analyses.

Testicular histology

Following fixation of testicular sections in 4% paraformaldehyde solution overnight, the fixed samples were transferred to a graded series of ethanol and xylene and then embedded in paraffin wax. Paraffin-embedded tissues were serially cut into 5-µm thick sections. Finally, two non-serial sections were stained with hematoxylin and eosin (H&E) using standard procedures for morphological analysis. Images were captured with a microscope (BX 51, Olympus) under 2X and 10X lenses using a video camera (XC 10, Olympus, Tokyo Japan).

Time-resolved fluoroimmunoassay (TR-FIA)

Concentrations of testosterone in serum were measured by a TR-FIA using the DELFIA Testosterone Kit (FI-20750 Turku, Finland) according to manufacturer protocols. The intra- and inter-assay coefficients of variation were <8.0%.

Immunohistochemistry

Following fixation of testicular sections in 4% paraformaldehyde solution overnight, the fixed samples were transferred to a graded series of ethanol and xylene and then embedded in paraffin wax. Paraffin-embedded tissues were serially cut into 5-µm thick sections onto 3-aminopropyl-triethoxysilane-coated slides (S8441, Matsunami, Tokyo, Japan). Briefly, sections were deparaffinized in xylene and rehydrated though a graded series of ethanol. To increase epitope exposure, the sections were heated in 0.01 M sodium citrate buffer, pH 6.0, at 121°C for 15 min in an autoclave. After incubating in 3% H_2O_2 (v/v) in methanol at 32°C for 30 min, the sections were blocked with normal goat serum for 1 h and then incubated overnight at 4°C with rabbit polyclonal antibodies specific to 17α-hydroxylase, diluted 1:1000 in 0.01 M phosphate-buffered saline, pH 7.2. Control sections were incubated with blocking serum alone. The specific protein immunoreactivity was visualized with the VECTASTAIN ABC Kit (Histofine, Tokyo, Japan) and a DAB kit (Histofine). In order to identify structural components and cell morphology, the sections were counterstained with hematoxylin and mounted with coverslips. Images were captured with a microscope (BX 51, Olympus) under a 20X lens using a video camera (XC 10, Olympus, Tokyo Japan).

Statistical analysis

Statistical analysis for testosterone levels was carried out with the GraphPad Prism version 5.0 statistical software program (GraphPad Software, San Diego, CA, USA).

RESULTS

Histological analysis

A gross evaluation of overall testicular architecture was undertaken using sections stained with H&E (Figures 1 and 2). Compared with diet and *ip* tBHQ-treated mice, mice treated without tBHQ showed more incompact interstitial components, and had higher germ cell loss in seminiferous tubules after scrotal heat exposure (Figure 1A-D). Mice treated with and without tBHQ by the *it* method both showed evident germ cell depletion and loose interstitial components in testes after scrotal heat exposure (Figure 1E and F, Figure 2E and F). Germ cell loss was also evident in testes of diet and *ip* tBHQ-treated mice (Figure 2B and D), even though the interstitial cells were more compact than those of mice treated without tBHQ (Figure 2A and C). In addition, deformed tubules were found in *ip* and *it* tBHQ-treated mice (Figure 2A and C).

Testosterone concentration

Differences in testosterone concentrations in serum among groups are shown in Figure 3. The average testosterone concentrations were 4.09 ng/mL (range 3.21-4.72 ng/mL) for diet-tBHQ (-), 0.21 ng/mL (range 0.15-0.30 ng/mL) for diet-tBHQ (+), 6.12 ng/mL (range 0.21-15.16 ng/mL) for *ip*-tBHQ (-), 0.34 ng/mL (range 0.06-0.81 ng/mL) for *ip*-tBHQ (+), 2.16 ng/mL (range 0.17-4.69 ng/mL) for *it*-tBHQ (-), and 0.41 ng/mL (range 0.07-0.71 ng/mL) for *it*-tBHQ (+). Scrotal heat mice from non-tBHQ groups had higher average testosterone

concentrations than those of normal, control mice (data not shown). Scrotal heated mice in all three tBHQ groups exhibited lower average testosterone concentrations than those in their corresponding non-tBHQ groups.

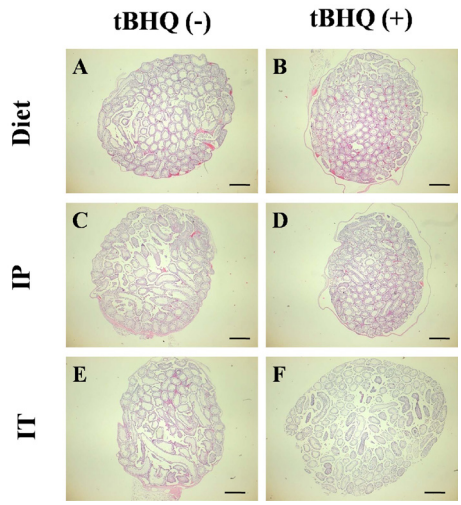

Figure 1. Cross-sections through whole testes from mice exposed to scrotal heat treated with or without *tert*-butylhydroquinone (tBHQ). **A.** and **B.** Mice administered with basal diet or tBHQ by diet way. **C.** and **D.** Mice administered with vehicle or tBHQ by intraperitoneal (*ip*) injection way. **E.** and **F.** Mice administered with vehicle or tBHQ by intratestis (*it*) injection. Scale bar = 500 µm.

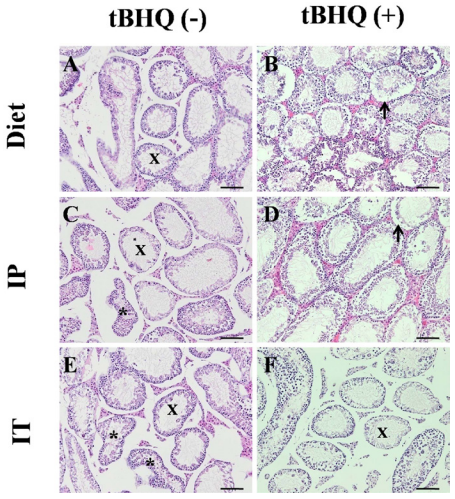

Figure 2. Histological evaluation of testes from mice exposed to scrotal heat treated with or without *tert*-butylhydroquinone (tBHQ). **A.** and **B.** Mice administered with basal diet or tBHQ by diet way. **C.** and **D.** Mice administered with vehicle or tBHQ by intraperitoneal (*ip*) injection way. **E.** and **F.** Mice administered with vehicle or tBHQ by intratestis (*it*) injection. x = highlighting tubules with depleted germ cells and collapse of the seminiferous epithelium; arrows = highlighting gaps and absence of spermatocytes in the seminiferous epithelium; asterisks = depressed tubules. Scale bar = 100 µm.

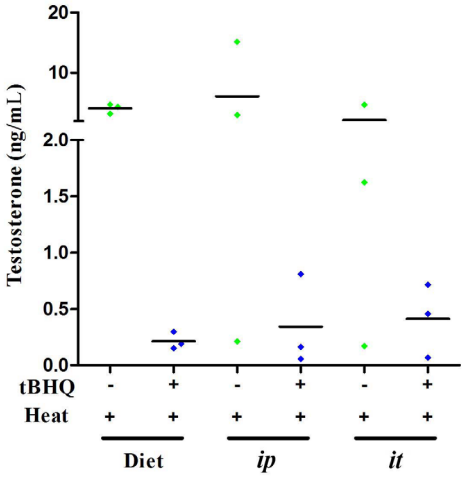

Figure 3. Testosterone concentrations in serum from mice exposed to scrotal heat treated with or without *tert*-butylhydroquinone (tBHQ). Short lines show average values for each group (N = 3 animals/group for scrotal heated groups (Diet, *ip* and *it*)). Points show the values for individual mice. Green squares denote the values for individual mice in non-tBHQ groups and blue squares denote the values for individual mice in tBHQ groups. *ip* = intraperitoneal; *it* = intratestis.

CYP 17 immunohistochemical analysis

The panels in Figure 4 illustrate representative immunohistochemistry images showing scrotal heat-induced alterations in CYP 17 protein expression in testes of the non-tBHQ and tBHQ groups. CYP 17 was only expressed in cells in the interstitial space. Cells in the interstitial space displayed higher CYP 17 expression in testes in non-tBHQ groups than in any of their corresponding tBHQ groups.

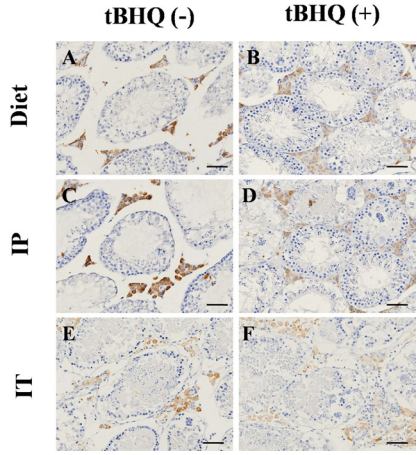

Figure 4. Immunohistochemistry illustrating alterations in CYP 17 protein expression in testes from mice exposed to scrotal heat treated with or without tBHQ. **A.** and **B.** Mice administered with basal diet or tBHQ by diet way. **C.** and **D.** Mice administered with vehicle or tBHQ by intraperitoneal (*ip*) injection way. **E.** and **F.** Mice administered with vehicle or tBHQ by intratestis (*it*) injection. The immunohistochemical signals appear brown and the counterstained background appears blue. Scale bar = 50 μm.

DISCUSSION

Impairments of the germ cell line induced by increased scrotal temperature are mainly associated with oxidative stress (Shiraishi, 2012). In the present study, we demonstrated that tBHQ has a protective effect on scrotal heat-induced damage in testicular architecture characterized by less germ cell loss and crowded interstitial cells in tBHQ-treated mice. The reduced testosterone concentration in serum and low CYP 17 expression indicated that tBHQ affected Leydig cells in the interstitial space. These effects may be related to the fact that tBHQ is an antioxidant compound and could improve the antioxidative capacity of interstitial cells.

An increase in scrotal temperature can cause the production of poor quality spermatozoa, germ cell depletion, and male infertility (Banks et al., 2005; Perez-Crespo et al., 2008). A previous study demonstrated a sharp reduction in testis weight and collapsed tubules in mice subjected to scrotal heat (42°C for 30 min), which resulted in abnormalities in embryonic development in female mice mated with heated male mice 23-28 days after treatment (Paul et al., 2008). Further studies reported that germ cell death was attributed to autophagy and oxidative stress-mediated apoptosis (Paul et al., 2009; Shiraishi et al., 2010; Zhang et al., 2012). In the present study, a single scrotal heat exposure (42°C for 25 min) induced germ cell loss in the seminiferous epithelium and incompact interstitial cells in mice testes, which were consistent with the previous reports mentioned above.

Many natural antioxidants can be effectively applied to eliminate oxidative stress by reducing the level of testicular-free radicals, including vitamin E (Cam et al., 2004), vitamin C, L-cysteine, and diphenyl-phenylene-diamine (Ahmed et al., 2011). In this study, we used tBHQ, which is not only a direct antioxidant used to preserve various oils, fats, and food by inhibiting autopolymerization of organic peroxides (de Guzman et al., 2009), but is also an indirect antioxidant acting as a nuclear factor erythroid 2-related factor 2 activator (Nrf2) to promote its accumulation in the nucleus and up-regulating the expression of antioxidant genes against oxidative stress (Cheung et al., 2011; Saykally et al., 2012). Several studies have explored the protective effect of tBHQ. Oral administration of tBHQ prevented glomerular injury in diabetic mice (Li et al., 2011). Moreover, pre-treatment with tBHQ also protected mice brains against traumatic brain injury-induced inflammatory damage (Jin et al., 2011). In the present study, deterioration of the seminiferous epithelium and loose interstitial cells were evident. tBHQ was applied as the antioxidant to counteract scrotal heat-induced damage in testes by both *ip* and *it* administration. With the exception of *it* administration, tBHQ was able to partly prevent scrotal heat-induced changes in testes, suggesting that it may function as an indirect antioxidant to induce Nrf2 accumulation and to up-regulate antioxidative enzymes against heat-induced oxidative stress.

Intratesticular testosterone plays a pivotal role in protecting germ cells against heat-induced cell death (Lue et al., 1999), while exogenous testosterone reduces intratesticular testosterone, resulting in germ cell apoptosis and suppression of spermatogenesis (Lue et al., 2000; Wang et al., 2007). Elevated scrotal temperature induced up-regulation of testosterone in patients with left varicocele, which might contribute to attenuate testicular oxidative stress-mediated apoptosis (Shiraishi et al., 2010). In the present study, compared with the basal testosterone concentration in serum (data not shown), the average values of testosterone in serum were all up-regulated in the non-tBHQ groups after scrotal heat exposure, implying that the steroidogenesis system up-regulated testosterone to prevent anabatic damage in response to heat-induced disorder in testes.

Testosterone synthesis depends on the concurrent action of several CYP enzymes, such as CYP 17 and 17β-hydroxysteroid dehydrogenase (Xiong and Hales, 1997). Inhibition of CYP 17, a key enzyme in androgen synthesis, suppressed testosterone synthesis in patients administered with abiraterone acetate (Attard et al., 2008). Our results showed a reduction of CYP 17 expression in interstitial cells of tBHQ groups, which might result in lower testosterone concentrations in serum than those of non-tBHQ groups. Steroidogenesis produces reactive oxygen species largely from mitochondrial respiration and the catalytic reactions of the steroidogenic CYP enzymes (Hales, 2002; Hanukoglu, 2006). In the average male, mitochondrial oxidative damage from steroidogenesis may be more of a chronic than an acute factor, and might be important in reducing testosterone production (Chen and Zirkin, 1999; Luo et al., 2006). A recent study reported that tBHQ could induce mitochondrial oxidative stress in HeLa cells (Imhoff and Hansen, 2010). In the present study, the reduced testosterone level and low expression of CYP 17 may have resulted from the pre-treatment of tBHQ, which could have induced chronic and mitochondrial oxidative stress, and thus decreased testosterone synthesis in testes. However, further studies are needed to elucidate the mechanism of how tBHQ affects steroid synthesis and prevents testicular heat-induced injury to maintain intact testicular architecture.

In summary, tBHQ might reduce testosterone concentration in serum via down-regulation of steroidogenesis. Considering the more intact testicular architecture, scrotal heat-induced changes were prevented by tBHQ treatment in testes after scrotal heat treatment. Therefore, tBHQ may be an optional antioxidant for heat-induced subfertility diagnosis.

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